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INTRODUCTION

Problem

Progesterone and estrogen are the main steroid hormones involved in breast development and tumorigenesis and can have both stimulatory and inhibitory effects on carcinogenesis that are both stage and dose dependent. The effects of these hormones are mediated through specific intracellular receptors. However, the specific contribution of these receptors to proliferation, differentiation and tumor growth of mammary tissue remains controversial. The overall objective of this project is to evaluate the physiological role of the progesterone receptor and its individual A and B isoforms in mammary gland development and tumorigenesis. Our approach is to examine the consequences of ablation of the PR A and B proteins on mammary gland physiology and function using PR null mutant mouse models.

Background

Progesterone and estrogen are the principle steroid hormones involved in normal breast development and tumorigenesis (1-3). In the case of mammary gland tumorigenesis, the effects of progesterone and estrogen on carcinogenesis can be both stimulatory and inhibitory and are dose and stage dependent (4). These hormonal effects are mediated by specific high affinity intracellular receptor proteins that are members of a superfamily of related transcription factors (5,6). Binding of steroids to these receptors results in the formation of activated receptor dimers that bind to specific enhancer DNA elements located in the promoter regions of hormone-responsive genes (7,8). The activation or repression of these genes represents the manifestation of the hormonal response.

The mammary gland is the site of milk production and secretion, and in females, is a major site of tumorigenesis (9). Mammary gland development occurs during the fetal, post-natal and adult stages of life (10). The development of the mammary gland occurs primarily post-natally and is directed by a complex signal transduction interplay between hormonal (polypeptide and steroid) and growth factor signals. During pregnancy, progesterone and estrogen promote growth and differentiation of normal mammary tissue by regulating ductal branching, alveolar formation (11) and lobuloalveolar development (12). Studies on the ontogeny of mouse mammary gland responsiveness to ovarian steroid hormones have indicated that receptors for estrogen and progesterone (ER and PR respectively) are present in both stromal and epithelial cells, and begin to exert effects on terminal end bud proliferation at 4 and 7 weeks of age, respectively (13). Furthermore, it now appears that epithelial cells, which can express receptors for estrogen and progesterone, are the major sites of primary mammary carcinomas (14).

Although the general consensus on progestin action in the uterus is that progesterone inhibits the proliferative effect of estrogen and acts as a differentiating hormone, this concept cannot be extended to the breast (3). Considerable evidence has accumulated to implicate progesterone in the proliferation of normal mammary epithelium in virgin animals (15) and in the development of the lobular-alveolar structure in mammary glands of pregnant animals (16). Unlike estrogen action, progesterone is a mitogen, not only in the epithelium of the terminal end buds, but also in the ductal epithelium (17). Depending on the time of administration and the dosage used, progestin agonists have been shown to reverse the anti-tumor effects of the anti-estrogen, tamoxifen, and induce tumor growth (18). The observation that the tumor inhibitory effect of tamoxifen can be reversed by

progestin agonists (18) together with the stage and dose dependent carcinogenic activity of progestin agonists (3) suggest that some of the effects of ERs may be mediated by PRs whose expression is known to be induced by estrogen (19). Taken collectively, the above data supports the proliferative effect of progesterone in normal breast development and in contributing to the oncogenic potential of the breast. Conversely, studies using the carcinogen-induced rat mammary tumor model (20) have shown that early pregnancy (21) or the administration of high doses of progesterone and 17 β estradiol (22) shortly after the onset of sexual maturity were effective in reducing the susceptibility of the mammary gland to chemical carcinogenesis. Thus, progesterone appears to have both stimulatory and inhibitory effects on mammary gland tumorigenesis that are stage and dose dependent.

From a clinical standpoint, the estrogen and progesterone receptor status of breast tumors is an important prognostic factor in determining the probability of disease free survival and response to hormonal therapy (2,23). Breast tumors that contain functional ERs and PRs have a higher response to hormonal therapy and higher disease free survival probability (2). However, as tumorigenesis progresses, the disease develops to a state that is characterized by a lack of ERs and PRs and a resistance toward hormonal and cytotoxic therapies.

It has been established that PR is composed of two hormone binding forms *in vivo*, termed PR_A and PR_B (24,25). It is thought that the A and B forms arise as a result of either alternate initiation of translation from a single mRNA (26) or by alternate transcription from promoters within the same gene (27). These receptor isoforms differ only in that PR_B contains an additional stretch of amino acids at the amino terminus of the receptor. Previous experiments have shown that these proteins exhibit different promoter specificities for target gene activation (28) while binding to the same enhancer DNA element (29). Remarkably, recent data have implicated a novel repressor function as well as an activator role for PR_A (30). Depending on the promoter and cell context, PR_A was shown to act as a potent transdominant repressor of PR_B-mediated gene transcription. In addition, the repressor function of PR_A was found to influence the activity of other members of this superfamily of transcription factors which included the glucocorticoid, mineralcorticoid and androgen receptors. Intriguingly, recent transient cotransfection experiments have revealed that PR_B when occupied by progestin antagonists can activate transcription (31). Furthermore, this unusual PR_B mediated antagonist transactivation can be dominantly inhibited by the PR_A isoform. This apparent paradoxical stimulatory action of progesterone antagonists via PR_B, if substantiated, would prompt a reevaluation or the potential efficacy of any chemoprevention strategy involving these 'anti-progestins' in the treatment of breast and uterine cancer.

Although, for two decades, the PR has been shown to be composed of two receptor isoforms, the specific physiological role for each of these two PR subtypes in normal breast development, tumor initiation and progression, has yet to be established. However, the existence of both these receptors in different species and tissues, and the elaborate mechanisms regulating their expression suggests that the absolute and relative levels (receptor status) of PR_A and PR_B in a progestin target cell are critical for the correct cellular response to progesterone and its antagonists. The equimolar expression of both forms of the PR in the same cell would allow the possible formation of two homodimers and one heterodimer (A:A, B:B and A:B). The potential existence of three dimeric forms of PR, each having different transcriptional regulatory specificities, would serve to further expand the repertoire of physiological responses to progesterone. Although breast tissue may contain an overall equimolar ratio of PR_A to PR_B, it is quite possible that different

cell types of this tissue, for example epithelial and stromal cells, may have a different ratio which is critical for the normal functioning of these cells. Therefore alterations in the ratio of PR_A to PR_B, would be expected to contribute to an altered susceptibility of these cells to carcinogenesis and have a dramatic effect on the cellular response to progesterone agonists, antagonists, other steroids and growth factors and proto-oncogenes regulated by progesterone.

An additional level of complexity in the involvement of these receptor isoforms in mammary gland development and tumorigenesis arises from influence of growth factors and proto-oncogenes such as epidermal growth factor (EGF), c-myc and cyclin D1 which have been shown to be increased by progestins in cultured human breast cancer cell lines (32). These mitogens may represent "early target" genes for progesterone which may act via autocrine and paracrine mechanisms to influence breast tissue proliferation and differentiation. At this stage, it is not known which of these gene products are modulated by either one or both isotypes of PR.

Purpose of the Present Work

Based on the above observation, we propose the following hypothesis:

During breast development and tumorigenesis, progesterone mediates its mitogenic effect through two receptor isoforms, PR_A and PR_B. We predict that, in vivo, PR_A and PR_B have distinct physiological effects and that the ratio of PR_A to PR_B is a key determining factor for normal breast development, oncogenic potential and carcinogenesis.

Methods of Approach

We have used a genetic approach to test the above hypothesis. Two fundamental questions regarding the role of progesterone and its receptor in breast development are being addressed. These are: (1) What is the *in vivo* functional significance of progesterone in general breast development? and (2) What is the *in vivo* functional relevance of the A and B forms of PR in normal breast development and tumorigenesis. These questions will be answered by the physiological analysis of mutant mice deficient in both forms of the receptor (PR_{A+B}-ve) and mouse lines deficient in either the A or B form of the receptor (PR_A-ve and PR_B-ve respectively). The generation of these mouse models will be accomplished by the mutation of the endogenous mouse PR gene by homologous recombination (gene targeting) in mouse embryonic stem (ES) cells. Pluripotent ES cells carrying the mutated PR allele will be injected into mouse blastocysts where they will become the progenitor cells of most of the embryonic tissues including the germ line. Germ line transmission of the mutated PR allele will allow the creation of mouse strains that are heterozygous and homozygous for the mutant PR gene.

Progress.

In the previous progress report, I described the phenotypic defects in mammary gland development that arise in null mutant mice lacking both forms of the progesterone receptor and provided evidence that the proliferative effects of PR in the mammary gland are mediated at least in part by PR dependent regulation of expression of the cell cycle protein D1 cyclin. I also reported the status of progress using two alternative strategies for introducing selective null mutations of the PR A or PR B proteins into the germline of mice. At that time, I reported lack of feasibility of one strategy

which was abandoned at some cost of time on the project and our generation of two new targeting constructs using a second alternative gene targeting strategy for producing the PRA and B mutations. Since our adoption of two targeting strategies caused some confusion to reviewers of that progress report, I will summarize the rationale and necessity at the time to try these approaches in this report. I will also detail the significant progress we have made in the past year **that has allowed us to obtain germ line mutation of both mutations in mice**. It is important to emphasise that the introduction of these mutations represents a significant technical challenge that is different from standard gene targeting approaches. The reasons for these difficulties will be detailed below. In fact our success in introducing these point mutations into the mouse genome this year is among the first to be achieved to date in mice for any gene.

Selective ablation of expression of the PR A or PR B protein expression in embryonic stem cells.

The A and B forms of PR arise from alternative initiation of translation at two different ATG codons on a single gene by alternative promoter usage. Thus, selective mutation of one of the two ATG codons will result in expression of a single form of the receptor. Our approach, therefore, to create mouse models that express only the A form of the receptor is to mutate the initiating ATG for PR B (ATG_B). Thus, the open reading frame for the PR B is destroyed. Similarly, mutation of the ATG site for PRA (ATG_A) will create a mouse model that expresses only the B form of the receptor. While the design of gene targeting constructs (see below) precludes us from testing these mutations in tissue culture cells, previous studies using cDNA expression vectors containing these mutations have demonstrated successful ablation of PRA or B expression when their respective ATG initiation sites are mutated (33).

Although over 200 genes have now been ablated in mice through gene targeting approaches, no reports to date have demonstrated successful gene targeting in mice by introducing point mutations into functional genes. This is primarily because selective markers used to select for uptake of targeting vectors must be removed in a relatively inefficient two step process before generating chimeric mice. Further, the two step procedures required for this targeting event can compromise the viability of embryonic stem cells and reduce their ability to transmit the mutation to the germ line. **Thus, our objective to introduce subtle mutations into the PR gene represented a technical challenge that is clearly more complex than our previous null mutation of the PR gene. We initially adopted two approaches to accomplish this goal.**

The first approach, which I proposed in my original application involved a two step homologous recombination strategy known as the "tag and exchange" approach that involved sequential use of two different gene targeting vectors. At the time of submission of the application, this approach had successfully been used by two groups to introduce subtle mutations into embryonic stem cells (34,35) but no demonstration of germ line transmission of these mutations to mice had been demonstrated. In the first step of this approach, a vector is constructed that contains two fragments of the mouse PR gene separated by two selectable markers (neomycin resistance gene and thymidine kinase gene) placed adjacent to each other. This vector is used to 'tag' the PR genomic locus by electroporation in embryonic stem cells and selection of homologous recombination events by G418 drug selection for neomycin resistance and southern analysis of resistant clones. The key difference between this vector and the standard gene targeting vector is that in the latter case, the TK gene is located outside the PR homologous gene fragments so that the TK sequences are lost upon

homologous integration and integrants can be selected for neo uptake and resistance using G418 and loss of TK by simultaneous selection with gancyclovir. In contrast, the TK sequences are retained in the tagging vector to allow a second independent selection event in the second step of recombination. The second targeting or 'exchange' vector simply contains a 5.0-7.0kb genomic fragment of PR containing the PR A or B point mutations. This vector is electroporated into isolated ES cell clones that contain the integrated tagging vector and used to replace the first vector by homologous recombination resulting in loss of both selectable markers allowing selection for TK loss using FIAU. The net result is that the endogenous gene is modified by a PR fragment that contains only the desired point mutation. Using this strategy, we successfully tagged the PR locus using a gene targeting vector that contained the neo and TK markers flanked by 1.2kb of PR sequence encoding part of exon 1 on the 5' side and 5.5kb of PR sequence encoding parts of exon 1 and 2 on the 3' side and achieved a 15% targeting frequency of integration of the tagging vector at the PR locus. However, as we reached this stage of the experiments, it became clear from studies in Dr. Alan Bradley's group and other collaborators at Baylor that although the second step of recombination is theoretically sound, in practice the FIAU selection step was yielding very poor selection efficiency and those rare targeted clones that were detected were losing viability due to extended manipulation and did not contribute to the germ line. For this reason, I considered that our likelihood of success in continuing with this approach was extremely low and decided that it was essential to adopt an alternative strategy that eliminated the necessity for the second homologous recombination step. The approach we adopted was the CRE-loxP system which relies on a highly efficient site specific recombination event in the second targeting step to remove markers and exogenous sequences from the PR gene locus. Although the change in strategy has cost us some time losses in generating new and different targeting vectors, the CRE-loxP strategy has proven highly successful and most importantly has allowed us obtain germ line transmission of a subtle mutation.

Mutagenesis Strategy.

The basic mutagenesis strategy was to use oligonucleotide directed mutagenesis to introduce nucleotide substitutions into the ATG codons that are responsible for initiation of translation of the A and B proteins of PR. An important issue to consider, in the case of the ATG_B initiation site, is that this region overlaps with a previously identified estrogen response element that may be important for estrogen dependent induction of PR expression. The mutagenesis strategy is therefore designed to avoid substitution of residues that may contribute to this regulation.

The basic approach was to introduce a conservative amino acid substitution at each initiation ATG together with an overlapping or nearby restriction site that would serve as a diagnostic site to allow easy detection of targeted events that contain the desired mutation. The mutations we introduced to selectively delete the A or B initiation ATGs are shown in Figure 1. In the case of the ATG_A mutation (panel A), we introduced a conservative substitution at the ATG changing MET to ALA (GCT) and a silent nucleotide substitution at the neighboring SER (AGT-AGC) to generate a novel NHE1 restriction site to facilitate detection of the mutant in embryonic stem cells by restriction analysis. In the case of the ATG_B mutation, we introduced a conservative substitution at the first nucleotide of the ATG changing MET to LEU so that the previously reported ERE consensus element was not disturbed and a silent mutation downstream of the ATG and consensus site to eliminate an endogenous PstI restriction site for detection of the mutation. Both mutations

were introduced into a 1.5kb genomic fragment of mouse PR encoding exon 1 using a Quick Change mutagenesis kit (Stratagene) and the individual mutations were sequenced before proceeding with generation of the targeting vector.

A. Wild type sequence

Ser Pro Leu *Met* Ser Arg Pro
TCC CCG CTC ATG AGT CGG CCA

Mutant sequence

Ser Pro Leu *Ala* Ser Arg Pro
TCC CCG CTC GCT AGC CGG CCA
NheI

B. Consensus ERE

Wild type sequence

G GTC ANN NTG ACC
Met Thr Glu Leu Gln
GG GTC GTC ATG ACT GAG CTG CAG
PstI

Mutant Sequence

Leu Thr Glu Leu Gln
TGG GTC GTC CTG ACT GAG CTC CAG

Figure 1. Comparison of the wild-type and mutant sequences in the region of the ATG translation initiation site for the A protein (panel A) and for the B protein (panel B).

Construction of mutant gene targeting vectors.

The CRE-lox P approach is a two step mutagenesis strategy that uses a modified PR targeting or tagging vector in which two selectable markers (neo and TK) are introduced into intron 2 of the mouse gene. The markers must be flanked by two lox P DNA sequences placed in the same orientation at the 5' and 3' ends of the markers. These sites act as specific recognition sequences for the site specific recombination enzyme, CRE recombinase which, when introduced into cells, will catalyse recombination between the two lox P sequences resulting in a deletion of the intervening marker sequences. Thus, in our case, step 1 involves electroporation of ES cells with a targeting vector that contains a PR genomic fragment containing the ATGA or ATGB point mutations and the lox P flanked markers in intron 2. Homologous integration of this vector into the endogenous PR locus serves to a) introduce the desired mutation and b) tag the PR genomic locus with markers that allow selection of homologous recombination events. Once ES cell clones have been identified that have integrated the targeting vector including the desired mutation at the PR locus, these clones are expanded and used for electroporation of a cmv-CRE recombinase expression plasmid to remove the marker sequences between the lox P sites.

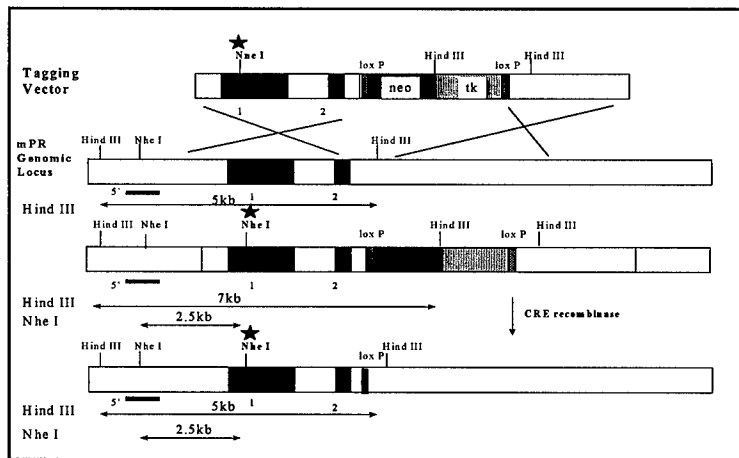


Figure 2. Gene targeting vector and strategy for generation of the ATGA mutation in embryonic stem cells.

Introduction of the ATGA mutation into ES cells.

The targeting vector and gene targeting strategy for introduction of the PR A mutation are shown in Figure 2. The vector contains (from 5' to 3' direction) 3.5kb of genomic PR sequence encoding exons 1-2 including the A initiation site mutations and part of intron 2 followed by the lox P flanked neo and tk selectable markers followed by an additional 3.0kb of PR genomic sequence encoding part of intron 2 at the 3' end. The vector was electroporated into ES cells and stable transformants containing the vector were selected by growth of the cells in the presence of G418 to generate neomycin resistant clones. Homologous integration into the PR locus results in a downstream shift of a unique HindIII site in intron 2 (due to the marker cassette insertion) resulting in the appearance of a 7.0kb hybridizing band representing the mutated allele by Southern analysis using a 0.5kb probe located upstream of the PR sequences used in the targeting vector and a 5.0kb band representing the wild-type allele.



Figure 3. Southern analysis of embryonic stem cells to detect homologous integration of the targeting vector containing the ATGA mutation at the PR locus. Homologous recombination events were detected by the appearance of a 7.0kb hybridizing band corresponding to the mutated PR allele in addition to the 5.0kb band representing wild type PR.

Using the above approach, we obtained a homologous gene targeting frequency of 12% of the screened neomycin resistant clones. An example of the Southern analysis of ES cell clones containing this vector is shown in Figure3. The results show that two of the clones screened on the filter contain both the 5.0kb (wild-type) and 7.0kb(mutant) alleles. Four independent targeted clones were selected for expansion and transfection with the CRE recombinase expression vector (pOG231, from Dr. S. O'Gorman, Salk Institute). After transfection the cells were grown in FIAU containing

medium to select for loss of the TK gene and screened again by Southern analysis to detect a) loss of the selectable markers, and b) presence of the ATG_A mutation (Figure 4). Removal of the selectable markers was detected using the same 5' probe to analyse Hind111 digested DNA to demonstrate loss of the 7.0kb band corresponding to the mutant gene with integrated markers with only the wild type 5.0kb band remaining. The ATGA mutation was then detected with the same probe by double digesting DNA with Hind111 and NheI. In this case, the wild type allele is represented by a 1.0kb 5' Hind111/NHE1 band and a 4.0kb 3' NHE1/Hind111 band and the mutant allele introduces an additional NHE1 site that results in a reduction in size of the 4.0kb band to 2.5kb while the 1.0kb 5' Hind111/NHE1 band is also detected. Of the four targeted clones analyzed, two contain the mutated allele at the ATG_A position. This result was not unexpected since during the initial homologous recombination event, cross over between the targeting vector and the endogenous PR gene may take place on either side of the point mutation in ATG_A resulting in a wild type or mutant product. Thus, we have two independent ES cell clones carrying the mutated ATG_A allele in the PR locus.

Generation of chimeric mice carrying the ATG_A mutation and identification of heterozygotes after germ line transmission of the mutation.

Both targeted clones have been microinjected into blastocysts from C57BL/6 female mice. To date we have successfully obtained 33 chimeric mice with more than 70% agouti coat color. Both clones have given rise to chimeras. Chimeric mice from each clone have been cross bred with wild type mating partners and both lines have given rise to germ line transmission of the ES cells. Screening of the agouti offspring from these matings has identified heterozygotes arising from the two independent ES cell clones. These animals were bred to maturity and intercrossed to detect homozygote offspring. An example of the Southern analysis of these offspring indicating 3 homozygotes in the samples analysed is shown in Figure 5. To date we have a total 8 homozygotes, 12 heterozygotes and 6 wild type mice from the first screening.

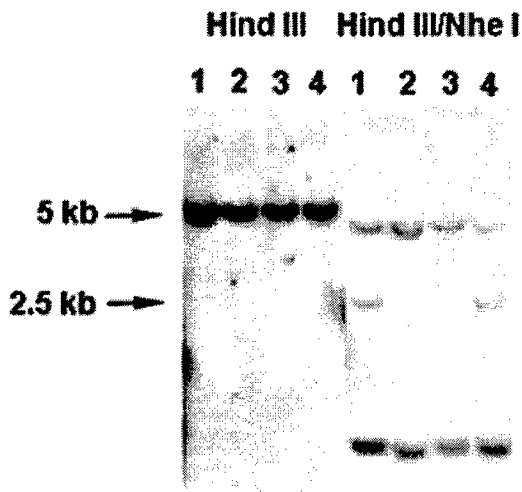


Figure 4. Detection of the ATGA mutation at the PR locus by Southern analysis of NHEI/HindIII digested E.S.cell DNA.

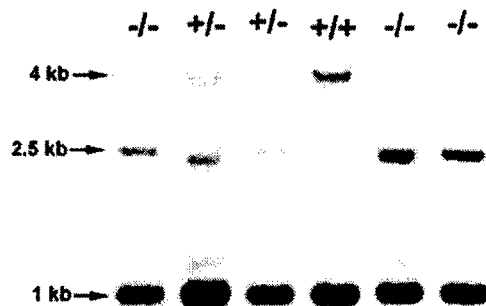


Figure 5. Confirmation of PR A null mutation (PR B+) using Southern hybridization analysis of Hind 111/Nhe 1 digested-DNA from offspring of heterozygous intercrosses.

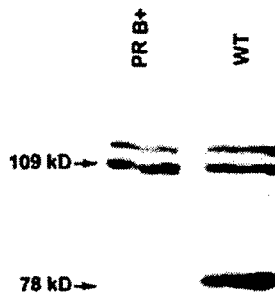


Figure 6. Western Immunoblot analysis of PR expression in uterus of wild type (WT) and PR A mutant mouse (PR B+) using rabbit anti PR IgG C-19 (Santa Cruz).

Western Analysis to confirm selective expression of the PR B protein in PR A null mutant mice.

To confirm exclusive expression of PR B in the homozygote mice, we treated 4 week old female homozygotes or wild type mice from the same litter with 10ug estradiol for 3 days and removed their uteri for Western analysis of PR expression. 100ug of uterine extract protein from each genotype was subjected to 6.5% SDS-PAGE electrophoresis and transferred to nitrocellulose. The PR isoforms were detected using C-19 rabbit anti-PR IgG (Santa Cruz Labs.), followed by HRP conjugated donkey anti-rabbit secondary antibody and ECL (Amersham) for visualization of the proteins. The results in Figure 6 clearly demonstrate that while a strong immunoreactive band corresponding to the A protein is detected in the wild type mouse, this band is eliminated from uteri of PRB+ mice. The B protein is detected at similar levels in both mice and is typically represented by one major and an upper minor band in mice that presumably results from phosphorylation of the

protein. Thus, we provide definitive evidence at the protein level to demonstrate selective ablation of expression of the PRA protein.

Introduction of the PR B mutation into embryonic stem cells.

The vector and gene targeting strategy for introduction of the PR B mutation into embryonic stem cells is shown in Figure 6. The overall vector design was essentially the same as that used for PRA except that the 3.5 kb PR genomic fragment located at the 5' side of the vector contained the ATG B mutation and deletion of the nearby PstI site shown in Figure 1. Southern analysis of HindIII digested DNA from ES cells electroporated with this vector (using the same approach as that described above) indicated that 10% of the G418 selected clones contained the 7.0kb hybridizing band corresponding to the mutated allele. Twelve of these targeted ES cell clones were expanded and electroporated with the CRE recombinase expression vector to remove the marker cassette from intron 2. After selection using FIAU, the clones were screened by PCR to confirm removal of the marker cassette and the PCR products were digested with PstI to identify clones that had lost the PstI site due to mutagenesis of the ATGB region. The oligonucleotides used were located 200bp upstream of the ATGB site and 400bp downstream of this site. Thus, the PCR product we predicted to obtain was 600bp which is not digestible with PstI in the case of the ATGB mutation, but is digested with PstI to yield a 200bp and 400bp fragment in the case of the wild type allele. The results in Figure 7 shows two of the screened clones contain the PstI mutation detected by the appearance of a 600bp PR fragment in addition the 400bp and 200bp fragments representing the wild type allele. PCR products from these clones were finally sequenced to confirm the presence of the ATG B mutation before electroporation of the targeted clones into blastocysts.

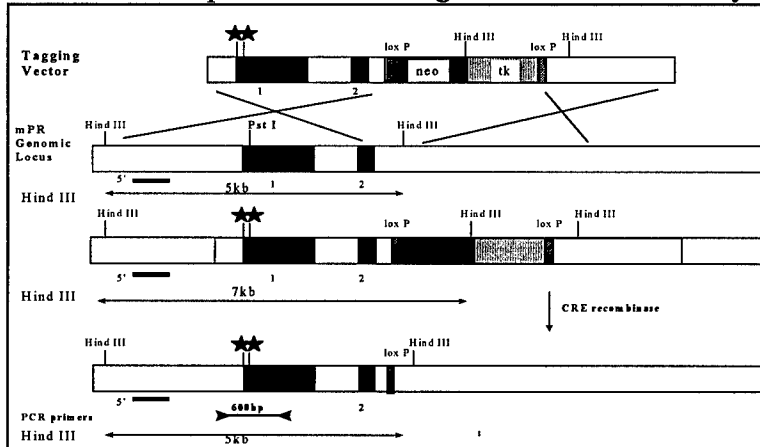


Figure 7. Targeting vector and strategy for introduction of the ATGB mutation into embryonic stem cells.

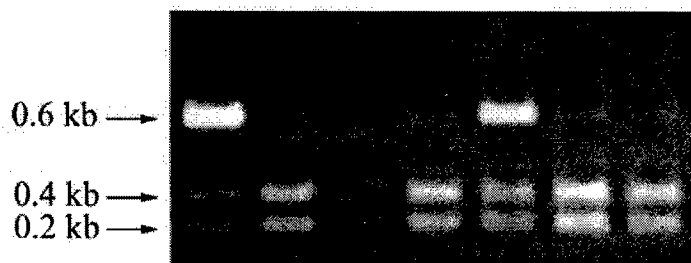


Figure 8. PCR analysis of targeted ES cell clones after loxP mediated recombination to detect homologous recombinants that contain the ATGB mutation using PstI digestion.

Generation of chimeric mice carrying the ATG B mutation.

Four targeted clones have been microinjected into blastocysts from C57BL/6 mice. We have generated 15 chimeric mice from two independent targeted ES cell clones. To date, cross-breeding of these mice has given rise to a total of 8 agouti coat colored offspring confirming germline transmission of the ES cells. While these mice have not been genotyped as yet, we are confident that 50% will carry a heterozygote PR B null mutation.

Conclusions. In summary, we have successfully targeted subtle mutations into the PR locus in embryonic stem cells to generate two novel mutant lines of mice that selectively inactivate expression of the A or B proteins of the PR. We have introduced these mutations into blastocysts to generate chimeric lines of mice carrying both mutations and we have confirmed germ line transmission of both mutations from chimeric mice and identified homozygote offspring from heterozygote mice in the case of the PRA mutation. During the next year, we will analyse the selective roles of the A and B proteins in mammary gland development. These mice will also provide a highly valuable tool to examine the selective responses of the A and B proteins to agonists and antagonists of the of PR in situ in the mammary gland.

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